

Sulfadoxine Resistance in *Plasmodium vivax* Is Associated with a Specific Amino Acid in Dihydropteroate Synthase at the Putative Sulfadoxine-Binding Site

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Sulfadoxine is predominantly used in combination with pyrimethamine, commonly known as Fansidar, for the treatment of *Plasmodium falciparum*. This combination is usually less effective against *Plasmodium vivax*, probably due to the innate refractoriness of parasites to the sulfadoxine component. To investigate this mechanism of resistance by *P. vivax* to sulfadoxine, we cloned and sequenced the *P. vivax dhps* (*pydhps*) gene. The protein sequence was determined, and three-dimensional homology models of dihydropteroate synthase (DHPS) from *P. vivax* as well as *P. falciparum* were created. The docking of sulfadoxine to the two DHPS models allowed us to compare contact residues in the putative sulfadoxine-binding site in both species. The predicted sulfadoxine-binding sites between the species differ by one residue, V585 in *P. vivax*, equivalent to A613 in *P. falciparum*. V585 in *P. vivax* is predicted by energy minimization to cause a reduction in binding of sulfadoxine to DHPS in *P. vivax* compared to *P. falciparum*. Sequencing *dhps* genes from a limited set of geographically different *P. vivax* isolates revealed that V585 was present in all of the samples, suggesting that V585 may be responsible for innate resistance of *P. vivax* to sulfadoxine. Additionally, amino acid mutations were observed in some *P. vivax* isolates in positions known to cause resistance in *P. falciparum*, suggesting that, as in *P. falciparum*, these mutations are responsible for acquired increases in resistance of *P. vivax* to sulfadoxine.

The rapid spread of chloroquine-resistant *Plasmodium falciparum* malaria in many countries has led to the widespread use of the sulfadoxine-pyrimethamine (S-P) combination, also known as Fansidar, for the treatment of malaria. Sulfa drugs (sulfones and sulfonamides) were first used in the 1930s for malaria treatment. Over the next decade, numerous clinical studies indicated that these drugs were usually effective against *P. falciparum* malaria but much less so against *Plasmodium vivax* malaria (13).

With the subsequent introduction of chloroquine and its ability to rapidly control acute attacks of both *P. falciparum* and *P. vivax* malaria, little further attention was given to the slower-acting sulfa drugs. However, when chloroquine resistance started to become a problem in the early 1960s, renewed interest was shown in sulfa drugs and in exploiting the synergy of these drugs in combination with dihydrofolate reductase inhibitors. Short-acting sulfonamides soon gave way to longer-acting sulfonamides such as sulfadoxine and sulfalene. As had been shown earlier with the shorter-acting sulfonamides (8, 19), the addition of pyrimethamine to sulfadoxine was appreciably more effective in curing chloroquine- and pyrimeth-

amine-resistant *P. falciparum* infections than sulfadoxine alone (3, 14).

The antibacterial and antimalarial modes of action of these sulfa drugs are quite similar in that they inhibit dihydropteroate synthase (DHPS), an enzyme that catalyses the condensation of *p*-aminobenzoic acid with 6-hydroxymethyl dihydropterin pyrophosphate to yield dihydropteroate (27). Sulfa drugs essentially block the *de novo* pathway of folate synthesis, leading to the depletion of dTTP and decreased DNA synthesis in both bacteria and malaria parasites. Since the sulfonamides and dihydrofolate reductase inhibitors, such as pyrimethamine, act at different points of the parasite's metabolic pathway, marked potentiation of antimalarial activity is usually observed with these drug combinations. The synergistic S-P combination is now used widely as a first-line drug in areas where *P. falciparum* has become resistant to chloroquine and pyrimethamine.

However, in most malarious countries located outside the African continent, *P. vivax* coexists with *P. falciparum*. Limited diagnostic facilities in many of these countries preclude a species diagnosis of malaria made upon microscopic examination of blood films. Since the two species cannot be differentiated on clinical grounds, malaria infections, whether due to *P. falciparum* or *P. vivax*, are treated with S-P. Unfortunately, S-P is less effective in clearing fever and parasites in patients infected with *P. vivax* than in those infected with *P. falciparum* (9, 11). Earlier studies had already shown that sulfa drugs are less

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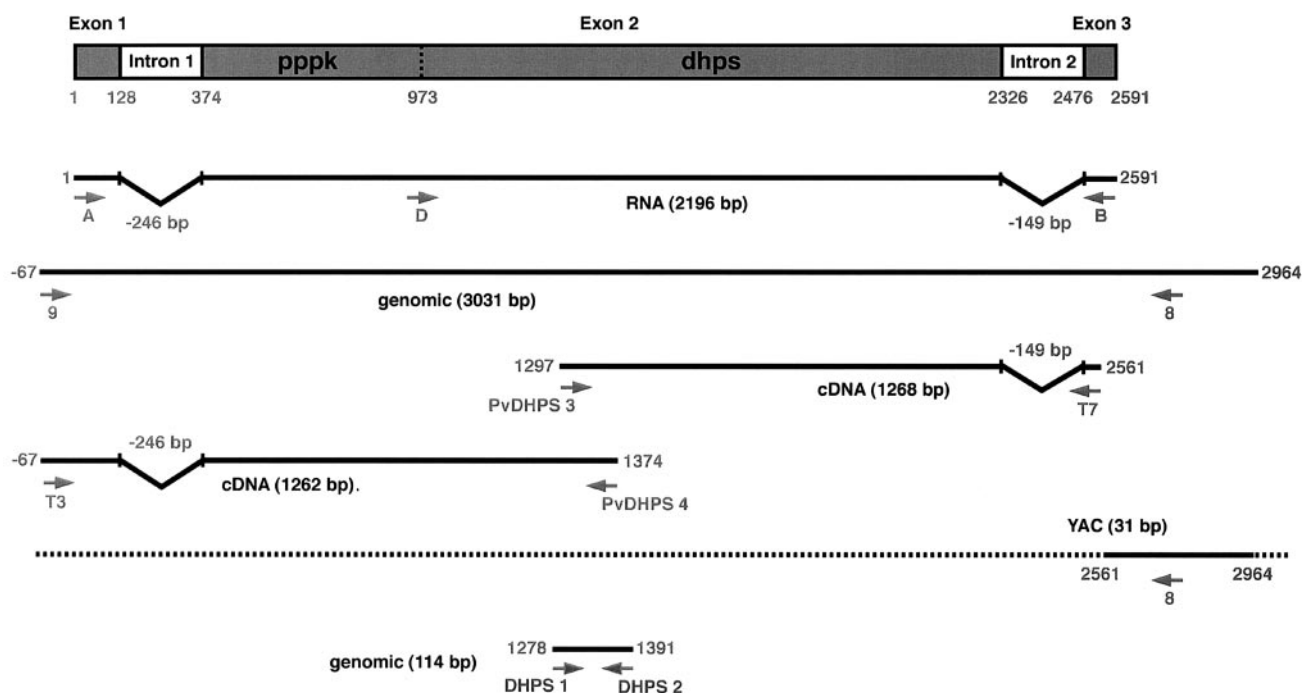


FIG. 1. Cloning strategy for the *pvpppk-dhps* gene. The expressed *pppk-dhps* sequence (exons) is represented by shaded boxes, and introns are represented by nonshaded boxes. A dotted vertical line indicates where the two coding regions join. Solid horizontal lines represent fragments of DNA or RNA sequenced to obtain the entire *pppk-dhps* gene sequence. A dip in the solid line represents a gap in the sequence. The dotted horizontal line indicates that the fragment is longer than shown. The numbering indicates distances in base pairs from the start codon. Numbers in brackets represent the sizes (in base pairs) of DNA or RNA fragments. Arrows indicate positions and orientations of primers.

active against *P. vivax* than against *P. falciparum* (16, 18, 20). Since there is already widespread resistance of *P. vivax* to pyrimethamine, the refractoriness of parasites to the sulfa drugs probably deprives the S-P combination of much of its synergistic activity when given to patients infected with *P. vivax* malaria.

The basis for the lack of response or innate resistance of *P. vivax* to sulfa drugs is not clear. However, it is highly probable that the mechanism involved in resistance of *P. vivax* to sulfadoxine is similar to that in *P. falciparum*. Sulfadoxine resistance in *P. falciparum* has been demonstrated to result from genetic mutations in the parasites' *dhps* gene (23, 26, 28). Several mutations in this gene resulted in increased 50% inhibitory concentration (IC_{50}) levels of *P. falciparum* to sulfadoxine. Among these, the A437G mutation has been shown by transfection experiments to be the initial mutation, causing a five-fold increase in IC_{50} . Higher IC_{50} levels were associated with the additional mutations S436F/A, K540E, A581G, and A613S (26). Therefore, sequencing the *P. vivax dhps* gene and developing an understanding of this resistance mechanism may help in formulating a better antifolate-sulfonamide combination that is effective against both *P. falciparum* and *P. vivax*.

In this paper, we report the cloning and sequencing of the *P. vivax ppvk-dhps* (*pvppvk-dhps*) gene and compare it to the *P. falciparum* homologue. Model three-dimensional structures of DHPS from *P. falciparum* and *P. vivax* derived from homology to other DHPS proteins were constructed, and the active sites were identified. Sulfadoxine was then docked into these

sites based on crystal structures of other DHPS proteins bound to similar sulfa drugs. The putative sulfadoxine-binding sites correlated with sequence polymorphisms associated with *P. falciparum* and *P. vivax* resistance. An explanation for the difference in binding of *P. falciparum* and *P. vivax* to sulfadoxine is presented, and the future role for sulfa drugs in combating malaria is discussed.

MATERIALS AND METHODS

Parasites. Several blood samples containing *P. vivax* were obtained from Australian travelers returning from Papua New Guinea (PNG) and East Timor and were frozen at -70°C . The patient blood was thawed, and genomic DNA was isolated by a method described elsewhere (7). Total RNA was isolated with the QIAGEN RNeasy kit. *P. vivax* samples from Maesod, Thailand (21), Fujian Province, China (1993), Morong, Philippines (1992), and West Papua (1999) (22) were kindly provided by B. Russell (AFRIMS, Bangkok, Thailand), Gao Qi (Jiangsu Institute of Parasitic Diseases, Wuxi, China), Cielo Pasay (Research Institute for Tropical Medicine, Manila, Philippines), and E. Tjitra (National Institute of Health Research and Development, Jakarta, Indonesia), respectively.

Cloning and sequencing the *P. vivax ppvk-dhps* gene. (i) **Amplification and sequencing of a genetic fragment conserved between species.** The *ppvk-dhps* genes of *P. falciparum*, *Escherichia coli*, *Toxoplasma gondii*, *Neisseria meningitidis* (GenBank accession numbers Z30654, X68777, U81497, and X68066, respectively), and *Pneumocystis carinii* (EMBL accession number U66282) were aligned, and the species conserved regions were identified. A pair of degenerate primers (DHPS1 and DHPS2), as shown in Fig. 1, were made to amplify a 114-bp fragment of the gene from a PNG isolate of *P. vivax* genomic DNA (Table 1). PCR was performed with 75 ng of each primer, 200 μM deoxynucleoside triphosphates, 2 mM MgCl_2 , 1 μl of genomic DNA, and 2.5 U of AmpliTaq Gold. Genomic DNA from *P. falciparum* and an uninfected human were used as positive and negative controls, respectively. The PCR was initiated by one cycle

TABLE 1. Primers used to amplify the entirety and fragments of the *P. vivax* *pppk-dhps* gene

Primer	Sequence (5'–3')
PvDHPS-1	CCTGAYTCYTTYTCVGAYG
PvDHPS-2	GGATTCGCCRCDDAYRTC
PvDHPS-3	GCGGTTTATTTGTCGATCC
PvDHPS-4	GTCAATCACGCTCGCC
PvDHPS-A	CCATGGAGGATTCAAACAC
PvDHPS-B	GAGATTACCTAAGGTTGATGTATC
PvDHPS-C	ATCATTAAGCATCGACACGGTC
PvDHPS-D	GGTTTATTTGTCGATCCTGTG
PvDHPS-8	TTGAAACACGCATTATGGTATCG
PvDHPS-9	AAGCGTAGCGACAGAAGAACG

at 94°C for 10 min followed by 45 cycles of 93°C for 50 s, 42°C for 50 s, and 72°C for 50 s. Sequencing this 114-bp fragment allowed the design of the *pvdhps*-specific primers PvDHPS3 and PvDHPS4.

(ii) **Screening *P. vivax* cDNA and YAC libraries and sequencing inserts of positive clones.** Four *P. vivax* cDNA libraries (K. Fischer, unpublished data) were constructed from patient-derived material (Belem, Brazil). PCR screening of cDNA libraries was achieved by using the vector specific primers T3 and T7 in combination with the *pvdhps*-specific primers PvDHPS4 and PvDHPS3, respectively (Fig. 1). The PCR products were sequenced directly. The *P. vivax* yeast artificial chromosome (YAC) library was constructed from *P. vivax*-infected erythrocytes from patients whom presented themselves to the Tropical Medical Centre in Rondonia, Porto Velho-Brazil, Brazil (5, 6). The YAC library was transferred to high-density nylon filters and screened by hybridization (6) with a *pvdhps* fragment obtained from a cDNA clone. A YAC clone containing the *pvdhps* gene was identified, prepared in agarose blocks, and separated by pulsed-field gel electrophoresis (5, 6). The agarose band containing the YAC DNA was isolated and digested with endonuclease as described by de Bruin et al. (10). The fragment containing the 3' end of *pvdhps* from the YAC insert was amplified by inverse PCR (25). Sequence tag sites were cloned into the pCR2.1 vector (Invitrogen) and sequenced.

(iii) **Amplification of the entire *pvpppk-dhps* gene from isolated parasite DNA and RNA.** Based on the DNA sequence obtained from the overlapping gene segments from the *P. vivax* YAC and cDNA libraries, several primers were designed to amplify the entire gene. Nested PCR was performed to amplify the full-length gene from genomic DNA with primers 9 and 8 as shown in Fig. 1 (10 min at 94°C, 50 s at 94°C, 50 s at 50°C, and 3 min at 72°C for 40 cycles) for first-round PCR and primers A and B (10 min at 94°C, 50 s at 94°C, 50 s at 50°C, and 3 min at 72°C for 40 cycles) for second-round PCR. The full-length coding region of the gene was obtained from total RNA by reverse transcription-PCR. This was carried out with the One-Step reverse transcription-PCR kit (Invitrogen) supplemented with *Pfu* by using primers 9 and 8 (Table 1) (30 min at 42°C, 2 min at 94°C, 50 s at 94°C, 50 s at 50°C, and 3 min at 72°C for 40 cycles) for first-round PCR, followed by second-round PCR with the Long PCR kit (Invitrogen) containing *Pfx* with primers A and B (2 min at 94°C, then 15 s at 94°C, 30 s at 55°C, and 3 min at 68°C for 35 cycles) (Table 1). PCR products amplified from both genomic DNA and total RNA were purified by using the QIAquick PCR purification kit (Qiagen) and then sequenced to identify introns and, hence, determine the full-length coding region.

Amplification and sequencing of the *dhps* portion from *P. vivax* isolates. The *dhps* portion of the gene from several *P. vivax* isolates was amplified by nested PCR. The first-round PCR was identical to that used to amplify the full-length gene, and the second-round PCR was performed with primers D and B to amplify only *pvdhps* (10 min at 94°C, 50 s at 94°C, 50 s at 50°C, and 1.5 min at 72°C for 40 cycles) (Table 1). The PCR product was purified and sequenced as described above.

Molecular modeling. (i) Homology models of *P. falciparum* and *P. vivax* DHPS. Homology models of DHPS from *P. falciparum* and *P. vivax* were constructed by using the homology package of InsightII from Molecular Simulations, Inc. The models were based on the DHPS crystal structures of *E. coli* (1AJ0) (1), *Thermoplasma acidophilum* (1PMA) (17), and *Mycobacterium tuberculosis* (1EYE) (2). The crystal structure template sequence segments of highest sequence identity and best secondary structure correlation were assigned to the DHPS sequences. Where the templates did not match the models, suitable loops with appropriate conformations were assigned from protein structures found in the Brookhaven PDB database. The models were minimized by using Discover

TABLE 2. Comparison of amino acid sequences of *Plasmodium* PPPK-DHPS

Organism	% Homology to:			
	<i>P. chabaudi</i>	<i>P. falciparum</i>	<i>P. vivax</i>	<i>P. yoelii</i>
<i>P. berghei</i>	88	58	56	82
<i>P. chabaudi</i>		60	56	78
<i>P. falciparum</i>			59	54
<i>P. vivax</i>				50

(Molecular Simulations, Inc.) to a root mean square deviation of less than 10^{-6} kcal/mol/Å by using steepest descents and conjugate gradients, taking into account morse and cross terms and charges. The DHPS models were validated by using Procheck.

(ii) **Docking of sulfadoxine and other sulfa compounds to the DHPS models.** Docking of the sulfa drugs to both *P. falciparum* and *P. vivax* DHPS models was performed by using genetic optimization of ligand binding from the Cambridge Crystallographic Data Centre.

(iii) **Predicted sulfadoxine-binding site.** Contact residues were defined as those that were found to be within 3 Å of the relevant sulfa compounds complexed to the corresponding *Plasmodium* DHPS models.

Nucleotide sequence accession number. DNA sequences reported in this paper have been deposited in GenBank under the accession number AY186730.

RESULTS

The *P. vivax* *pppk-dhps* gene. A region of genomic DNA covering 3,031 bp of the entire *pvpppk-dhps* gene was sequenced. Putative start and stop codons from within the *pvpppk-dhps* gene were determined by comparison with the *P. falciparum* *pppk-dhps* gene. The entire gene contains 2,591 bp (Fig. 1) and two introns that were identified by comparing sequences of genomic DNA and total RNA. Intron 1 is 246 bp in length (nucleotides 128 to 373) and intron 2 is 149 bp in length (nucleotides 2327 to 2475). The coding region of the gene contains 2,196 bp encoding 731 amino acids.

Sequence homology between the *P. vivax* *pppk-dhps* gene and other *Plasmodium* *pppk-dhps* genes. The DNA and putative amino acid sequences from the *pvpppk-dhps* gene were aligned and compared to sequences of other *Plasmodium* species located in the GenBank database. It was found that, at the amino acid level, *P. vivax* PPPK-DHPS has 56, 56, 59, and 50% homology to PPPK-DHPS from *P. berghei*, *P. chabaudi*, *P. falciparum*, and *P. yoelii*, respectively (Table 2). The *pppk-dhps* sequences are highly homologous between different species of malaria parasites; however, a repetitive domain that was not found in the other malaria parasites sequences exists at the *pvpppk-dhps* C-terminal region. This domain is composed of 7-amino-acid tandem repeats.

Mutations in the *P. vivax* *dhps* gene. The *dhps* portion of the *pvpppk-dhps* gene was amplified and sequenced from 14 isolates with geographically diverse backgrounds (Table 3). A sequence comparison revealed only two amino acid changes among the *P. vivax* isolates: A383G in 4 isolates from Thailand and 2 isolates from West Papua and A553G in 4 Thai isolates, equivalent to A347G and A581G in *P. falciparum* DHPS, respectively. Amino acids in *P. vivax* DHPS at positions corresponding to those in *P. falciparum* DHPS where mutations were reported are shown in Table 3. The amino acid at position 585 in all of the *P. vivax* DHPS sequences contained a Val

TABLE 3. Amino acid changes observed in *P. vivax* DHPS genes in several isolates

Allelic type	Origin (no. of isolates)	Amino acid at positions ^a :				
		436, 382	437, 383	540, 512	581, 553	613, 585
Wild	East Timor (1), Brazil (1), West Papua (1), China (2), Philippines (1), PNG (1)	S	A	K	A	V
Mutant	West Papua (2), Philippines (1)	S	G	K	A	V
Mutant	Thailand (4)	S	G	K	G	V

^a The first position listed is for *P. falciparum*, and the second position listed is for *P. vivax*.

compared with an Ala in wild-type *P. falciparum* DHPS sequences.

***P. vivax* and *P. falciparum* DHPS homology models.** Homology models of *P. vivax* and *P. falciparum* DHPS were constructed based on the deduced crystal structures of DHPS from *E. coli* (1AJ0), *T. acidophilum* (1PMA), and *M. tuberculosis* (1EYE). The amino acid sequence alignment and structurally conserved region (SCR) elements of DHPS from *E. coli* DHPS

(1AJ0) and the two models are shown in Fig. 2. The SCRs predicted in both the *P. falciparum* and *P. vivax* DHPS models were comparable to those reported in the three template structures, with the exception of one omission of a large stretch of amino acids in *P. falciparum* (positions 620 to 656) and the omission of two large stretches of amino acids in *P. vivax* (positions 411 to 438 and 592 to 681). These amino acids could not have a structural orientation assigned to them due to the lack of homology with the crystal structure templates. However, these omissions are unlikely to affect the active sites or the predictions made from the resultant homology models.

The predicted sulfadoxine-binding site in *P. vivax* DHPS differs from the equivalent site in *P. falciparum* by one amino acid. Sulfadoxine was docked into the predicted active site in the *P. falciparum* DHPS model as well as the equivalent active site in the *P. vivax* DHPS model. The docking was guided by the orientation of the sulfa drug sulfanilamide in complex with DHPS from *E. coli* (1AJ0). Multiple energy minimizations were performed, yielding an energy minima and a location most representative of the binding site (Fig. 3A). A total of 10 contact residues were predicted to directly interact with sulfadoxine (Table 4). The *P. falciparum* predicted sulfadoxine-

<i>Pvi</i>	ERTHIVGI	LVNVDYDFSD	GGLFVDPVKA	VERMFEMASD	GASVIDIGGE	[380]
<i>Pfa</i>	EKTNIWGI	LVNVDYDFSD	GGIFVEPKRA	VQRMFEMINE	GASVIDIGGE	[434]
<i>Eco</i>	MKLFAQTSL	DLSPHVMGI	LVNTPDSFSD	GGTHNSLIDA	VKHANLMINA	GATIIDVGGG [60]
<i>Pvi</i>	SSAPYVVPNP	SVTERDLVMP	VLKLFKEEWH	KLECEVGGGA	VCCAAASDAR	KNAQSSQLQK [440]
<i>Pfa</i>	SSAPFVIPNP	KISERDLVVP	VLQLFQKEWN	D-----	-----IKN	[468]
<i>Eco</i>	STRPGAAEVS	VEEELQRVIP	VVEAIAQRFE	V-----	-----	[91]
(omitted from model)						
<i>Pvi</i>	LQKVRDAKPI	ISIDTVNYDL	FKECVELGVL	DILNDISACT	HNPEIIKLLR	RKNKFYSVVL [500]
<i>Pfa</i>	KIVKCDKAPI	ISIDTINYNV	FKECDVNDLV	DILNDISACT	NNPEIIKLLK	KKNKFYSVVL [528]
<i>Eco</i>	-----W	ISVDTSKPEV	IRESAKVG-A	HIINDIRSL	EP--GALEAA	AET-GLPVCL [138]
<i>Pvi</i>	MHKRGNPHTM	DKLTNYDDLI	SDIKRYLEDR	LHFLVLNGVP	RYRVLFVGL	GFAKKHDQSI [560]
<i>Pfa</i>	MHKRGNPHTM	DKLTNYDNLV	YDIKNYLEQR	LNFLVLNGIP	RYRILFDIGL	GFAKKHDQSI [588]
<i>Eco</i>	MHMQGNPKTM	QEAPKYDDVF	AEVNRYFIEQ	IARCEQAGIA	KEKLLDPGF	GFGKNLSHNY [198]
<i>Pvi</i>	KLLQHIHVDY	E--YPLFLGY	SRKRFTVHCM	GKGGXAIGEC	RLMSGKALT	NGEGKLTNGE [618]
<i>Pfa</i>	KLLQHIHVDY	E--YPLFIGY	SRKRFTVHCM	NDQ-----	-----	[619]
<i>Eco</i>	SLLARLAEFH	HFNLPLLVGM	SRKSMIGQLL	N-----	-----	[229]
<i>Pvi</i>	AKLTNGEGKL	TNGEAKLTNG	EGKLTNGDAK	LTNGDSKLTN	GEAKLTNGDP	SQLWRFKMSH [678]
<i>Pfa</i>	-----	-----	-----NVVI	NTQQLHDEQ	QENKNIVDK	SHNWMFQMNY [653]
<i>Eco</i>	-----	-----	-----	-----	-----	[229]
(omitted from model)						
<i>Pvi</i>	MRQDKDQLLY	QKNICGGLAI	ASYSFYKKVD	LIRVHDVLXT	KAVLDVLTRI	HQP [731]
<i>Pfa</i>	MRQDKDQLLY	QKNICGGLAI	ASYSFYKKVD	LIRVHDVLET	KSVLDVLTKI	DQV [706]
<i>Eco</i>	-----VGP	SERLSGSLAC	AVIAAMQGAH	IIRVHDVKET	VEAMRVVEAT	LSAKENKRYE [282]

FIG. 2. Amino acid sequence alignment of DHPS from *P. vivax* (*Pvi*), *P. falciparum* (*Pfa*), and *E. coli* (*Eco*). SCR elements are represented by grey shading. Residues putatively involved in sulfadoxine binding are shown in bold, and residues known to be associated with mutations that lead to sulfadoxine resistance in *P. falciparum* are boxed. The residue V585 from DHPS in *P. vivax*, predicted to be responsible for innate resistance to sulfadoxine, is circled.

TABLE 4. Predicted sulfadoxine contact residues in *P. falciparum* and *P. vivax* DHPS

Amino acid position no. in <i>P. falciparum</i>	Amino acid in:		Amino acid position no. in <i>P. vivax</i>
	<i>P. falciparum</i>	<i>P. vivax</i>	
436	S	S	382
437	A	A	383
438	P	P	384
580	F	F	552
608	R	R	580
609	K	K	581
613	A	V	585
663	Y	Y	688
666	N	N	691
688	H	H	713

binding site contained an A613 residue, whereas the *P. vivax* sulfadoxine-binding site contained a V585 residue at the equivalent position. This was the only difference between the predicted sites of both models for sulfadoxine binding (Fig. 3A and Table 4).

Mutations in DHPS from *P. falciparum* associated with resistance to sulfadoxine are located in or near the *P. falciparum* DHPS sulfadoxine-binding site. The mutations S436F/S436A, A437G, K540E, A581G, and A613S shown in Fig. 3B that are known to cause resistance by *P. falciparum* to sulfadoxine were separately introduced into the *P. falciparum* DHPS model. The model was energy minimized for each mutant, and from these results, predictions were made. The replacement of Ser with Phe at position 436 is a major alteration not allowing sulfadoxine to bind due to the bulky ring causing a massive steric hindrance. A putative hydrogen bond from S436 to sulfadoxine or S436 to a putative sulfate ion would also be destroyed, destabilizing that region of the binding site. Replacing the same Ser with an Ala would have a much less serious effect with little steric hindrance at all. An Ala-to-Gly change at position 437 would result in a loss of hydrophobic interaction between the Ala side chain and sulfadoxine ring, significantly reducing the binding affinity (Fig. 4A). K540 is a surface residue located approximately 10 Å away from the sulfadoxine-binding site. A K540E change at this position results in a positive-to-negative charge reversal. This in turn reorients R610 and, hence, the contact residues P438 and K609, causing a moderate change to the binding site (Fig. 3B). Similarly, A581 is approximately 9 Å from the sulfadoxine-binding site. However, it resides in a turn that gains increased flexibility when A581 is replaced with Gly, causing disturbance to the contact residue F580 but only a very minor disturbance to the binding site (Fig. 3B). The Ala-to-Ser mutation at position 613, although subtle, would also result in a reduction in hydrophobic contacts between itself and sulfadoxine coupled with the introduction of some polarity through the lone pairs on the serine side chain oxygen (Fig. 3B).

V585 in *P. vivax* DHPS is predicted to disrupt the sulfadoxine-binding site. Energy minimization of the *P. falciparum* DHPS sulfadoxine and *P. vivax* DHPS sulfadoxine complexes revealed an increase in energy in the *P. vivax* DHPS sulfadoxine complex caused by steric hindrance from the larger V585 residue compared with the smaller A613 residue in the *P.*

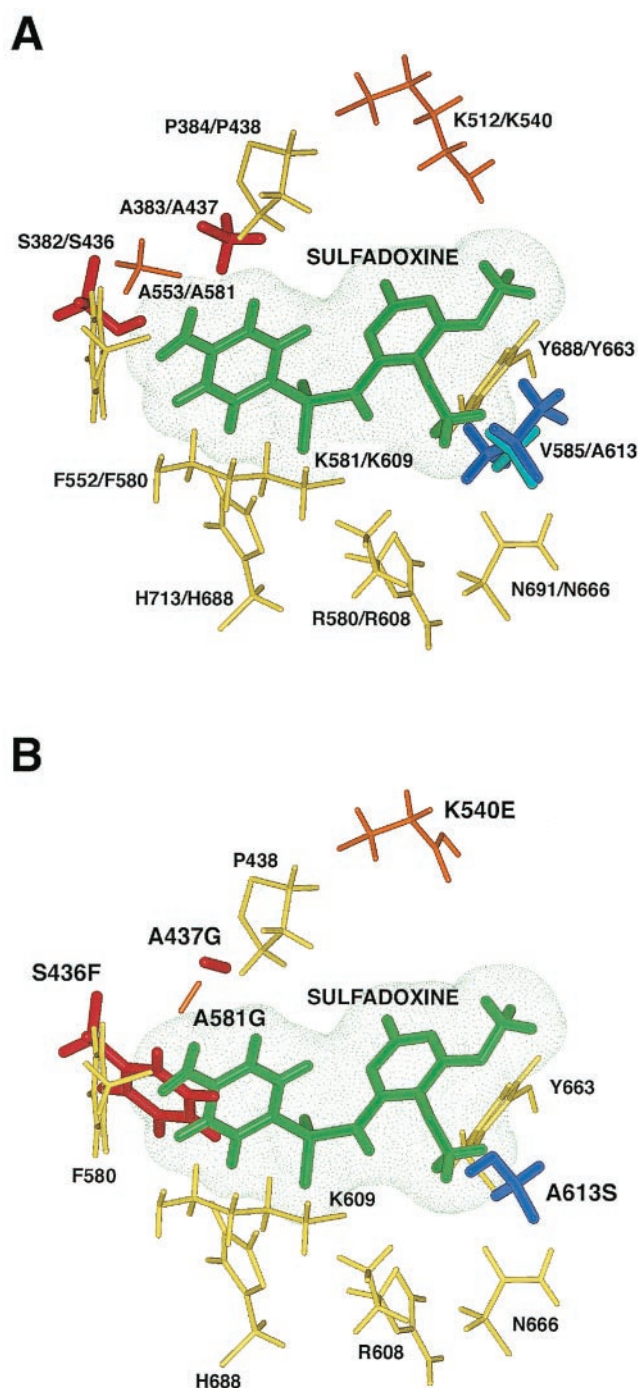


FIG. 3. (A) Stick representation overlay of the putative sulfadoxine-binding sites from *P. falciparum* and *P. vivax* DHPS. Sulfadoxine is shown in green stick form with its electron densities as small dots. All amino acids shown in thick (contact residue associated with resistance) or thin stick form are predicted contact residues with the exception of K540/K512 and A581/A553. S436/S382 (red), A437/A383 (red), A613/V585 (blue), K540/K512 (orange), and A581/A553 (orange) are all associated with *P. falciparum* resistance to sulfadoxine. Innate resistance to sulfadoxine in *P. vivax* is predicted to be a consequence of the V585 residue. (B) Stick representation of the putative binding site in DHPS from *P. falciparum* for sulfadoxine. The mutations S436F, A437G, and A613S associated with resistance by *P. falciparum* to sulfadoxine have been shown to demonstrate their potential effects on the binding site.

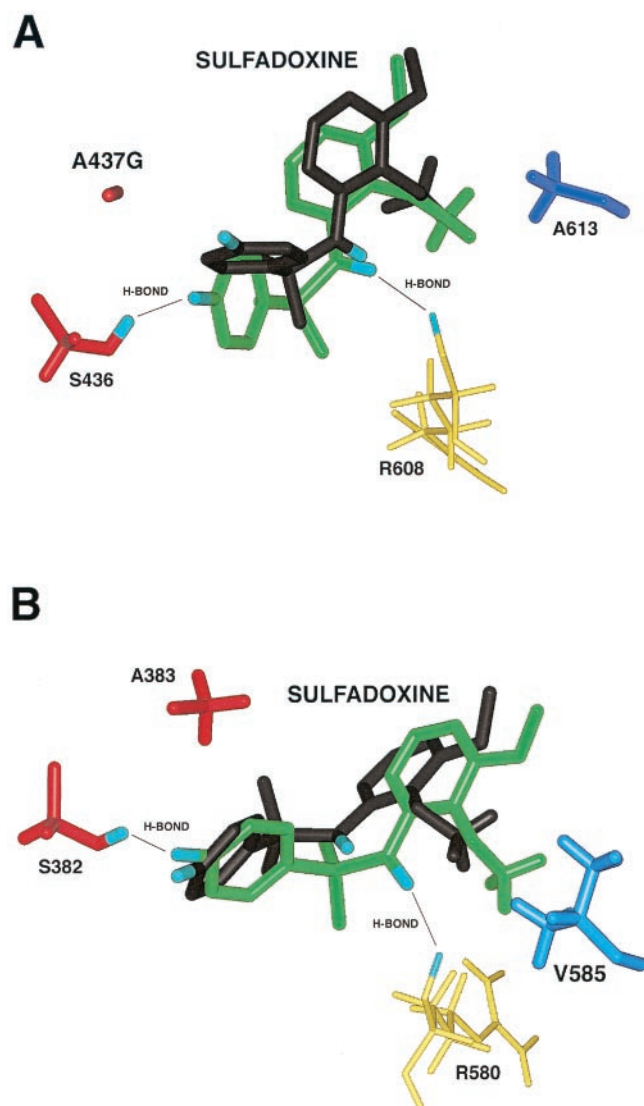


FIG. 4. (A) Effect of the A437G mutation in DHPS from *P. falciparum*. The equivalent residues from Fig. 3A are shown here for *P. falciparum* with the exception of residue A613 (dark blue). There is a loss of hydrophobic interactions between the A437G residue and sulfadoxine (colored as described for Fig. 3A). There is a reduction in binding of sulfadoxine to DHPS from *P. falciparum* when the A437G mutation is present. (B) Illustration of the effect of V585 at the DHPS *P. vivax* binding site compared with A613 in *P. falciparum* DHPS. *P. vivax* residues prior to energy minimization are displayed in stick form and colored red and blue. Sulfadoxine is also shown in green stick form in the equivalent position it adopted in the *P. falciparum* binding site and in black at the position adopted after energy minimization, allowing for the effect of V585. Hydrogen bond donor and acceptor atoms are colored light blue and are broken after energy minimization. The V585 interferes with binding of sulfadoxine to DHPS from *P. vivax*.

falciparum DHPS sulfadoxine complex (Fig. 3A). The larger side chain of the valine caused a steric hindrance in this region of the binding site compared with alanine, resulting in the separation of the two predicted hydrogen bonds from S382 and R580 to sulfadoxine (Fig. 4B).

Effect of mutations in the *P. vivax* DHPS protein on sulfadoxine binding. Two mutations were identified from sequencing the *dhps* portion of the *pvpppk-dhps* genes from 14 *P. vivax* isolates with diverse geographical backgrounds: A383G and A553G, equivalent to A437G and A581G, respectively, in *P. falciparum* (Table 3). The modeling predicts that the disruption that these two mutations cause in *P. vivax* to the sulfadoxine-binding site is similar to that of *P. falciparum* (Fig. 4A and B).

Binding interactions of other sulfa drugs to the *P. falciparum* and *P. vivax* sulfadoxine-binding site. Sulfadimethoxine, sulfamethoxypyridazine, sulfadiazine, sulfalene, sulfathiazole, sulfamethoxazole, sulfafurazol, dapsone, and acedapsone were energy minimized into the *P. falciparum* and *P. vivax* sulfa drug binding sites to determine from a molecular level whether these drugs may be used as components of future drug regimens. It was predicted that V585 would interfere with the binding of sulfadoxine, sulfadimethoxine, sulfamethoxypyridazine, and sulfalene to DHPS from *P. vivax* (data not shown). In contrast, it was predicted that the binding of sulfadiazine, sulfathiazole, sulfamethoxazole, sulfafurazole, dapsone (Fig. 5), and acedapsone to DHPS from *P. vivax* would not be affected by the presence of V585. The model was also used to investigate the effect that the A383G mutation in *P. vivax* DHPS isolates would have in the binding of other sulfa drugs. Unfortunately, it was predicted that this change would significantly reduce the binding of all of the aforementioned sulfa drugs to DHPS from *P. vivax*,

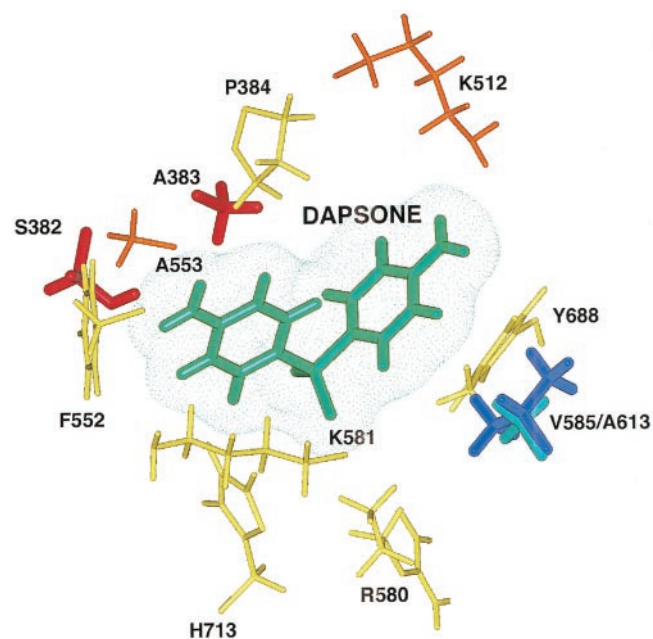


FIG. 5. Stick representation of the putative binding site in DHPS from *P. vivax* for dapsone. Dapsone is shown in fluorescent green stick form with its electron densities as small dots. Residues are colored and rendered as described for Fig. 3, with the exception of position V585. The equivalent residue A613 in DHPS from *P. falciparum* and residue V585 have been overlaid to show that dapsone does not interact directly with either residue. Dapsone binding to DHPS from *P. vivax* is not predicted to be hindered by the presence of V585.

given that both classes contain very similar core elements within their structures and that this mutation occurs at such a region.

DISCUSSION

Unlike most antimalarial drugs, sulfadoxine is innately less effective against asexual erythrocytic parasites of *P. vivax* than *P. falciparum*. In Technical Report Series 711, the World Health Organization recommended that antifolate-sulfonamide combinations should not be used for the therapy of *P. vivax* infections because of widespread resistance to antifolates and the inadequate efficacy of sulfonamides against this parasite (29). To determine the molecular mechanism causing the inadequate efficacy of sulfonamides against *P. vivax*, we have cloned and sequenced the *pppk-dhps* gene in *P. vivax* and investigated the putative sulfadoxine-binding sites in DHPS from *P. falciparum* and *P. vivax*.

The *dhps* gene of *P. vivax*, as in *P. falciparum*, is connected to the *pppk* gene, which codes for a bifunctional enzyme. The protein sequence of *P. vivax* DHPS is moderately homologous to those of other malaria species; however, sections of the protein are more homologous to other malaria species and to *E. coli*. This made it possible for homology models of *P. falciparum* and *P. vivax* DHPS to be constructed based on the *E. coli* DHPS crystal structure. The models allowed a putative binding site for sulfadoxine in both *P. vivax* and *P. falciparum* to be identified based on the crystal structures of *E. coli* DHPS complexed to sulfanilamide and the use of energy minimization.

The *P. falciparum* DHPS model was first utilized to help understand the effects that known mutations in DHPS from *P. falciparum* have on sulfadoxine binding. Predictions from energy minimizations were compared with transfection results published by Triglia et al. (26), where various *P. falciparum* DHPS mutant alleles were transfected to replace the wild-type *P. falciparum dhps* gene and sulfa drug IC₅₀s of the mutant combinations determined. The mutations S436F, S436A, A437G, and A613S associated with sulfadoxine resistance in *P. falciparum* were all found to be located within 3 Å of the *P. falciparum* DHPS sulfadoxine-binding site. These mutant complexes, when energy minimized, resulted in higher energies and, hence, lower binding capacities to sulfadoxine, consistent with significant increases in IC₅₀s obtained in the transfection experiment (26).

The two other mutations studied, A581G and K540E, are not predicted to be in direct contact with sulfadoxine in the *P. falciparum* DHPS sulfadoxine-binding site. The mutation A581G only resulted in a slight increase in energy when introduced into the complex. It resides approximately 9 Å from the binding site, causing minor secondary effects through other contact residues. The prediction is consistent with a slight increase in its IC₅₀ in the transfection experiment (26). The K540E mutation is also approximately 10 Å from the binding site but results in a side chain charge reversal from positive to negative. This mutation alone resulted in a larger increase in energy that is consistent with the additional increase in IC₅₀ when this mutation was compared in combination with and without other mutations in the transfection experiment (26). It should be noted here that the

K540E mutation is not predicted to cause treatment failure when found independent of other mutations. The K540E mutation is primarily found in combination with up to 5 additional mutations in DHPS and dihydrofolate reductase (4, 12, 15, 24). It is most likely that K540E correlates well with treatment failure due to its combination with other mutations in these genes.

The *P. vivax* homology model of DHPS was then used to analyze the effect of V585 in comparison to A613 in DHPS from *P. falciparum*. Energy minimization of sulfadoxine bound independently to DHPS from both *P. vivax* and *P. falciparum* resulted in a higher energy for the *P. vivax* complex due to the added bulk of the valine side chain interfering with sulfadoxine. This result is supported experimentally by the existence of an A613S mutation in *P. falciparum* (equivalent to position 585 in *P. vivax*) that has been shown to be associated with sulfadoxine resistance (26). This, coupled with the result that all the other putative binding residues were identical between the two species, suggests that it is highly probable that V585 is largely responsible for *P. vivax* resistance to sulfadoxine.

V585 is the only amino acid that is different between the *P. falciparum* and *P. vivax* predicted sulfadoxine-binding sites and is present in the 14 geographically diverse *P. vivax* isolates sequenced so far. We therefore hypothesize that the existence of V585 is possibly a wild-type residue, thus providing a logical explanation for the observed innate resistance of *P. vivax* to sulfadoxine. It is an early speculation that requires more DHPS sequencing data from *P. vivax* isolates from various geographical settings, especially from areas with limited S-P usage, to confirm this hypothesis.

In contrast to the apparent sequence conservation of the V585 residue, an A383G mutation was detected in some isolates of *P. vivax*. It was predicted, by use of energy minimization, that the A383G mutation would cause a reduction in binding of sulfadoxine to *P. vivax* DHPS. This same change has also been found to be essential for conferring sulfadoxine resistance in *P. falciparum*. Since it is only found in some *P. vivax* isolates, it may represent the acquired resistance that developed as a result of a relatively recent selection event due to the widespread intensive use of sulfadoxine and other sulfa drugs. In the limited number of sequencing experiments that we performed, this mutation is most prevalent in isolates from Thailand, which is consistent with high resistance levels observed for *P. falciparum* in the area, suggesting a high selection pressure on both species of *Plasmodium*.

The construction of these DHPS models also made it possible to test whether other sulfa drugs could be predicted to be more useful than sulfadoxine against *P. vivax*. In particular, the testing of dapsone has significant implications due to the recent development of a new sulfa drug-dihydrofolate reductase inhibitor combination comprised of dapsone and chloro-proguanil, which will soon replace S-P. Interestingly, dapsone is much smaller than sulfadoxine and is not predicted to bind directly to V585. Energy minimization of the *P. vivax* DHPS dapsone complex with V585 yielded no significant energy difference compared to the same complex energy minimized with A585, the equivalent amino acid observed in *P. falciparum* DHPS. Unfortunately though, unlike V585, the A383G muta-

tion is predicted to affect all sulfa drugs that can potentially bind to *P. vivax* consistent with the cross-resistance profile for three sulfonamides and a sulfone demonstrated by Triglia et al. (24). These predictions may provide an explanation for the variable effect of dapsone in the treatment of *P. vivax* patients (18, 20). That is, dapsone may be effective in treating a patient infected with a V585 *P. vivax* isolate but most probably fails when a patient is infected with a V585/A383G *P. vivax* isolate.

Due to the difficulties involved in culturing the *P. vivax* parasite, the in vivo effect of having V585, A383G, and A553G in *P. vivax* is difficult to demonstrate. The identification and similarities of the sulfadoxine-binding sites in DHPS from both *P. falciparum* and *P. vivax* suggest that A383G and A553G in *P. vivax* DHPS will result in similar increases in resistance to sulfadoxine in *P. falciparum*. It may be inferred that this is the case, given that Russell et al. (21) have shown that four of the Thai isolates (V585/A383G/A553G) presented in this paper are resistant to sulfadoxine (MICs, 500,000 ng/ml).

The identification of V585 in DHPS from *P. vivax* as well as homology modeling of DHPS itself has made it possible to predict that the V585 residue is most probably responsible for resistance to sulfadoxine by *P. vivax* and is potentially the underlying cause of innate resistance. It is also predicted by modeling that this residue would not affect the sensitivity of *P. vivax* to dapsone and several other potential sulfa drugs. Unfortunately though, the wide use of sulfa drugs in the treatment of bacterial and plasmodium infections may have contributed to the selection of *P. vivax* populations with the mutations A383G and/or A553G. The presence of mutations affecting the sulfa drug binding site common to most sulfa drugs may render dapsone, and other sulfa drugs not predicted to be affected by V585, useless for the treatment of *P. vivax*. This situation and the spread of chloroquine resistance in *P. vivax* emphasizes the urgent need to develop alternative drugs and drug strategies for effectively treating both *P. falciparum* and *P. vivax*.

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